UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Review of the Zhao *et al.*, 2020 study on "Genetic breakdown of a Tet-off conditional lethality system for insect population control" and its relevance to the OX5034 *Ae. aegypti* Experimental Use Permit; EPA File Symbol 93167-EUP-1.

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BACKGROUND

On November 15, 2020, the Florida Keys Environmental Coalition (FKEC) informally submitted to EPA an article by Zhao and colleagues, which evaluates the frequency of the genetic breakdown (resistance) of a single lethal trait in the fruit fly *Drosophila melanogaster* (Zhao *et al.*, 2020). FKEC raised concerns that these results would similarly indicate survival of OX5034 females (Oxitec Ltd.) in the environment. In its risk assessment for OX5034 *Ae. aegypti*, EPA considered several scenarios that could result in the survival of female mosquitoes, including genetic resistance, and found their likelihood to be negligible in the context of the Experimental Use Permit (EUP).

Although the topic of genetic resistance was discussed in the risk assessment, the Zhao *et al.*, 2020 article itself was not considered as part of the risk assessment as it was published after the EUP was issued. We note that the Zhao study references a similar Oxitec product (OX513A), which unlike OX5034 showed some female survival. EPA evaluates the study in the context of the EUP for OX5034 and its implications for female survival and determines that the conclusion from the risk assessment remains unchanged.

OX5034 *Ae. aegypti* are mosquitoes that were genetically engineered such that a single lethal gene is selectively expressed in females that carry the OX5034 gene cassette. With continued releases of OX5034 males, the *Ae. aegypti* population in that area is expected to decline over the course of several mosquito generations due to a lack of viable female offspring sufficient to sustain the population. On April 30, 2020, EPA issued an EUP for OX5034 to allow for field testing over two consecutive years at specific sites in Florida and Texas (EPA File Symbol 93167-EUP-1).

OX5034 contains the active ingredient tetracycline-repressible transactivator protein variant tTAV-OX5034 protein, the inert ingredient DsRed2-OX5034, and the genetic material (vector pOX5034) necessary for their production in OX5034 *Aedes aegypti*. OX5034 female lethality is attributed to the overexpression of the tTAV-OX5034 protein in immature females, a process that is thought to interfere with the transcriptional machinery of the insect and consequently normal cellular function. As a result, homo- and hemizygous females carrying the OX5034 genetic cassette survive only until the early larval stages (L2/L3). Overexpression of *tTAV-OX5034* is achieved through a gene circuit that is based on the "Tet-OFF" system, which was originally described in *E. coli,* and that produces a variant of the tTA protein (Gossen and Bujard 1992). In *Ae. aegypti*, basal *tTAV-OX5034* expression is enhanced through a positive feedback loop (i.e., expression of tTAV-OX5034 promotes expression of more tTAV-OX5034) that can be suppressed through the addition of tetracyclines to the larval diet. The inert ingredient DsRed2-OX5034 is located on the same genetic cassette as tTAV-OX5034 and used as a visual marker in the laboratory to identify field-collected *Ae. aegypti* that carry the OX5034 traits.

STUDY SUMMARY

The goal of the Zhao study was to determine the frequency with which heritable genetic resistance occurs in a single-trait self-limiting system. To that end, the group developed a non-sex-specific homozygous *D. melanogaster* line using a modified variant of the *head involution defective* (hid^{Ala5}) gene to effect lethality through programmed cell death (Grether *et al.*, 1995; Horn and Wimmer, 2003; Table 1). A modified variant of HID was used in this study to prevent downregulation of the protein by the Ras signaling pathways; a mechanism that would reduce lethality (Bergmann *et al.*, 1998). Expression of hid^{Ala5} is activated by the presence of tTA, which is encoded on a second cassette that was also integrated in the fruit fly genome. Unlike OX5034, where tTAV-OX5034 is involved in a positive feedback loop, the system in the flies relies on a linear, unidirectional system where tTA promotes expression of hid^{Ala5}

and HID^{Ala5} leads to lethality. tTA itself is under the control of an enhancer-promoter from the serendipity α cellularization gene (*s1*), which initiates the expression of tTA (and thus *hid*^{Ala5}) during the embryonic stages of fruit fly development in both males and females (Horn and Wimmer, 2003). Like tTAV-OX5034 in *Ae. aegypti*, tTA expression in the fruit fly can be stopped through the addition of tetracycline in the rearing medium. Because the gene coding for tTA (driving *hid*^{Ala5} expression) and *hid*^{Ala5} were introduced into the fruit fly genome on separate cassettes, each cassette also carried a visually distinguishable marker, ECFP on the *hid*^{Ala5} cassette and DsRed on the tTA cassette. These markers allowed for tracing of these components in the fruit fly line, which is important as both cassettes are needed for lethality to occur.

To determine the frequency with which mutations lead to offspring survival, male fruit flies homozygous for both genetic cassettes were reared in the presence of tetracycline and crossed with wild-type females. 1.2 million heterozygous offspring were then screened in the absence of tetracycline for incidences of survival. A total of 109 individual F_1 progeny (0.0091%) survived, of which 20 (0.0017%) were subsequently able to produce viable offspring. The surviving F_2 were then inbred and individuals sequenced to identify mutations that rendered the lethality system ineffective. Sequencing revealed a total of 7 primary-site mutations, which are mutations within the inserted cassettes, and 13 second-site mutations, which were hypothesized to be maternal effect variations that either suppressed or effected the function of the *hid*^{Ala5} gene, i.e., those that are specific to the function of HID^{Ala5} in this transgenic line, and that are found outside of the of the inserted cassettes. Table 1 lists notable similarities and differences between the fruit fly line and OX5034.

Table 1. Comparison between the *D. melanogaster* (Zhao *et al.*, 2020) and OX5034 lines

	D. melanogaster	**OX5034**
Lethal trait	HID^{Ala5}	tTAV-OX5034
Sex-specific	No. Both male and females affected.	Yes. Only females affected.
Enhancer	tTA	tTAV-OX5034 (tTA variant)
Enhanced expression of lethal trait	No.	Yes. tTAV-OX5034 expression leads to increased tTAV-OX5034 production though a positive feedback loop.
Number of cassettes	Two. Both genetically linked and located on chromosome 2.	One.
Visual markers	Two. ECFP for tTA and DsRed for *hid*^{Ala5}	One. DsRed2-OX5034 for tTAV-OX5034

RELEVANCE TO THE CURRENT OX5034 EUP

Spontaneous mutations naturally occur in all organisms and they contribute to the genetic diversity within a species. However, the frequency with which spontaneous mutations in OX5034 are expected to result in genetic changes that would allow for female survival in the absence of tetracycline to occur is very low. The Zhao et al. study confirmed this expectation in fruit flies, finding that out of 1.2 million flies carrying a conditional lethal trait only 0.0091% survived and 0.0017% were subsequently able to pass the mutation to their offspring.

There are several factors that contribute to this low likelihood of female survival through genetic breakdown. First, mutations are not confined to the genes coding for or otherwise affecting the components of a lethality system and mutations anywhere else in the genome are not expected to affect the lethality of the self-limiting gene. However, even if a mutation were to occur within the genetic

components of the lethality system, not every mutation is expected to be meaningful because not every mutation is expected to affect the function of the lethality system. For example, some mutations may be silent in that they do not change the amino acid sequence of the lethal protein, and yet others may occur in parts of the gene that are unlikely to negatively affect the function of the lethal trait, such as small substitutions within linker sequences. Likewise, if a mutation were to affect the production of a lethal trait, even if that reduction is substantial and extends longevity, it does not necessarily mean that it also allows for survival into adulthood and/or successful reproduction. This is because expression of lower amounts of the lethal trait may still result in a general fitness burden. Secondly, for a mutation to allow for survival, changes must occur in a genetic component that is integral to the function of the lethal trait without also affecting other essential cellular functions. For example, mutations in the pre-mRNA splicing complex would likely reduce lethality caused by the lethal protein, but at the same time negatively affect the transcriptional machinery as a whole. As such, these mutations would carry a significant fitness cost for the individual, ultimately reducing and not increasing its chances for survival. Taken together, it is not surprising that in the pool of 1.2 million hemizygous fruit flies, Zhao and colleagues found that of the few individuals that survived in the F_1, less than one fifth, i.e., 20, were found to carry heritable mutations.

In addition to the factors provided above, there are specific considerations that are relevant for the evaluation of the Zhao et al. study in the context of the OX5034 EUP. Zhao et al. developed the fruit fly line specifically to determine the frequency of genetic breakdown of the lethal tTA/HID^{Ala5} system. To accomplish that, transgenic flies needed to be reared under optimal conditions in the laboratory in order to remove environmental variables that could affect their survival. In contrast, OX5034 will be released into the field and under these conditions, survival is expected to be lower. Further, based on the number of nucleotides composing the lethality system, the fruit fly line has a statistically greater chance to acquire mutations as it has two large genetic cassettes. This consequently provides increased opportunity for mutation to occur compared to a single, smaller construct in OX5034. Therefore, while the Zhao et al. study found a low frequency of genetic resistance in the transgenic fruit fly line, in OX5034 it is expected to be even lower.

In addition to the expectations based on population genetic theory, data evaluated by EPA as part of the risk assessment show that female lethality of the OX5034 sex-specific lethal trait is 100% in OX5034 homo-and hemizygous individuals of the corresponding wild-type background as well as in hemizygous individuals resulting from matings with mosquitoes with a genetically distinct background from field trials in Brazil (U.S. EPA, 2020). Further, genetic resistance to the OX5034 trait has not been observed in 27 generation equivalents of OX5034, nor as part of the field releases involving over 12 million OX5034 homozygous males.

CONCLUSION

The Zhao et al. study is consistent with EPA's assessment that there is a negligible potential for OX5034 female survival during the EUP due to genetic resistance to the lethal trait. Demonstration of offspring survival in the Zhao study does not imply that there will be survival of female OX5034 during the EUP. Therefore, this study does not alter EPA's risk assessment for OX5034. As EPA recognized spontaneous mutations as a potential avenue for resistance development, it required Oxitec to continually monitor for the presence of resistance in field-collected hemizygous *Ae. aegypti* larvae for the duration of the EUP. Based on the product-specific data evaluated by EPA, the Agency finds that instances of female survival into adulthood due to genetic resistance are expected to be negligible within the parameters of this EUP.

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